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Journal of Chromatography A, 828 (1998) 135–140

JOURNAL OF CHROMATOGRAPHY A

On-line deconjugation of chloramphenicol-β-D-glucuronide on an immobilized β-glucuronidase column Application to the direct analysis of urine samples

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Abstract

An immobilized HPLC column has been developed for the on-line deconjugation of β-glucuronides. The enzymatic activity of this column has been previously demonstrated [1]. This study reports on the application of the immobilized β-glucuronidase column to the analysis of glucuronide metabolites in the urine. The system utilized in this work was composed of an internal-surface reversed-phase (ISRP) column (50×4.6 mm) containing a hydrophobic inner phase and a hydrophilic outer phase, a β-glucuronidase immobilized enzyme reactor (BG-IMER) column (50×4.6 mm) and a C_8 reversed-phase column (150×4.6 mm). The columns were connected with three six-port switching valves. A coupled-column procedure was developed for urine samples containing chloramphenicol-β-D-glucuronides (0.07–1.1 m*M*/injection). Urine samples were injected into the ISRP column where the glucuronides were separated from the biological matrix, with matrix contaminants eluting off-line to waste. Eluent from the ISRP column containing the glucuronides was then transferred on-line to the β-glucuronidase column for deconjugation and passed directly on-line to the C_8 column. In this portion of the chromatographic procedure, the mobile phase consisted of 0.01 *M* ammonium acetate at pH 6.7. The analyte concentrated on the top of the reversed-phase column was then eluted using a gradient mobile phase system of acetonitrile and 0.01 *M* ammonium acetate (pH 5.0) and detected at UV wavelength of 280 nm.

Keywords: Stationary phases, LC; Column switching; Chloramphenicol-glucuronide; Antiobiotics

1. Introduction

Chloramphenicol (Fig. 1) is an antibiotic isolated by Bartz in 1947 [2] from a culture of *Streptomyces venezuelae*. Until recently, chloramphenicol was a drug of first choice to treat severe gram-positive and -negative infections. Because of its potential to cause severe toxic reactions, chloramphenicol usage is now limited to serious infections in which safer alternatives are not available. It is a drug that is therefore especially useful in meningitis patients who are allergic to penicillin derivatives [2].

Chloramphenicol is mainly metabolized in the liver and only 5–10% of a dose is excreted as unchanged drug in the urine [3]. Chloramphenicol is primarily conjugated with glucuronic acid but also undergoes oxidative and reductive pathways [4].

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0021-9673/98/$ – see front matter
PII: S0021-9673(98)00815-2

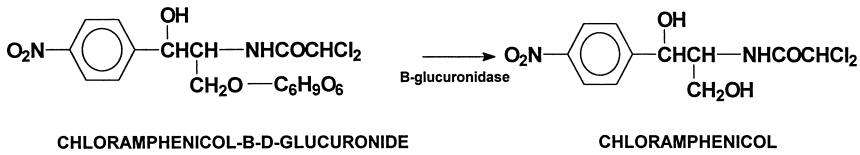

Fig. 1. Molecular structures of chloramphenicol and its glucuronide metabolite.

Most metabolites, including chloramphenicol-β-D-glucuronide, are excreted in the urine [3] but a small proportion may be eliminated in the bile. Gut β-glucuronidase enzymes (lysosomal acid hydrolases) may therefore deconjugate a small percentage of this glucuronide metabolite.

Most investigators currently identify and quantify the total concentration of glucuronide metabolites using β-glucuronidase enzymes in incubation assays [5,6]. We recently proposed the use of a novel β-glucuronidase column (BG-IMER) for this purpose [1]. The activity and stability of the BG-IMER column was previously reported on a broad range of substrates [1]. The BG-IMER column has been found to be stable for at least 8 weeks (0.01 *M* ammonium acetate, pH 5.0) without significant changes in the activity [1].

The objective of the current study was to develop a coupled-column HPLC method for determining urinary concentrations of hydrolyzed chloramphenicol-β-D-glucuronide using the BG-IMER enzyme column.

2. Experimental procedure

2.1. Equipment

The HPLC system consisted of two solvent delivery pumps (Thermo Separations Products San Jose, CA, USA), a Model p1000 single solvent pump and a binary solvent pump Model p2000; a UV Spectra 100 variable-wavelength detector and a Chromjet integrator. A Rheodyne injector, Model 7125 (Rheodyne, Cotati, CA, USA) with a 100-μl loop was used for sample injection. Three Rheodyne switching valves (Model 7010) were used for on-line connections between the two systems.

2.2. Columns

Three analytical columns were used in this study. The first column (Column A) was an ISRP (internal-surface reversed-phase column from first generation of packings [7]), 5.0 cm×4.6 mm I.D., stainless steel column (phase GFFII) packed with 100-Å pore size and 5-μm particle size silica purchased from Regis (Morton Grove, IL, USA).

The second column (Column B) used in the study was a BG-IMER (β-glucuronidase) enzyme column synthesized according to previously described procedures [1]. The β-glucuronidase enzymes (isolated from *E. coli* K12, RNase negative) were purchased from Boehringer Mannheim (Laval, Canada), and covalently immobilized on a 300-Å, 5-μm silica support (Kromasil 200, Eka Chemical, Bohus, Sweden) packed in a 50×4.6-mm stainless steel column.

The third column (Column C) was used to analyze the products (concentrated on the packing bed of the column) obtained from the BG-IMER column. This was a Rexchrome C_8 (100-Å pore size, 5-μm particle size, 150×4.6 mm I.D) analytical column purchased from Regis.

2.3. Chemicals and reagents

The BG-IMER column was stored at room temperature in a solution that had been slightly modified from what was previously reported [1]. The storage solution consisted of 0.01 *M* phosphate buffer (pH 7.0), 0.01% sodium azide and 10% glycerol. The mobile phases consisted of (A) HPLC-grade acetonitrile and (B) 0.01 *M* ammonium acetate buffer (HPLC grade) dissolved in filtered water (Millipore water filtering apparatus; Millipore, Bedford, MA, USA), adjusted to pH 5.0 with acetic acid, filtered through a 0.2-μm nylon filter (WSC, Ottawa,

Canada) and degassed under nitrogen for at least 30 min. These chemicals were all purchased from Moquin Scientific (Quebec, Canada). Chloramphenicol and its glucuronide metabolite were purchased from Sigma (Oakville, Canada). Human urine was used as the biological matrix.

2.4. Preparation of standards

Stock solutions of chloramphenicol and its glucuronide metabolite (chloramphenicol-β-D-glucuronide) were prepared by dissolving the appropriate amounts of each substrate in filtered water to yield a final drug concentration of 15.5 and 18.4 m*M*, respectively.

A calibration curve was constructed by spiking concentrations of chloramphenicol-β-D-glucuronide into 200 μl of urine. A concentration range of 0.07–1.1 m*M* was injected (100 μl) into the ISRP column. Blank urine specimens were used as negative controls while samples containing the parent drug, chloramphenicol spiked in urine, were used as positive controls. All urine samples were centrifuged at 12 000 *g* for 5 min prior to injection.

2.5. Chromatographic procedure

Fig. 2 demonstrates a schematic diagram of the coupled HPLC system. The following experimental steps were followed:

The system used three six-port valves to direct the sample flow between the three HPLC columns. A 100-μl sample of urine (centrifuged 5 min at 12 000 *g*) containing a concentration range of chloramphenicol-β-D-glucuronide (0.07–1.1 m*M*) was injected directly into column A and urine components were eluted off-line to waste for 5 min. The elution mobile phase used was composed of 0.01 *M* ammonium acetate (pH 6.7) and run at 0.25 ml/min using pump 1. The glucuronides were eluted on-line through Column B (to be hydrolyzed) and the aglycone was then concentrated on the column bed of Column C. The system was switched to pump 2 to analyze the product, chloramphenicol (aglycone). The final chromatography was performed on Column C with mobile phases consisting of acetonitrile (mobile phase A) and 0.01 *M* ammonium acetate (pH 5.0) (mobile phase B). A gradient system running initially at a flow-rate of 0.25 ml/min for 2 min (100% mobile phase B), was increased to 1.0 ml/min (100% mobile phase B), followed at 32 min of a mixture of mobile phases A–B (50:50). The procedure was performed at ambient temperature with UV detection at 280 nm.

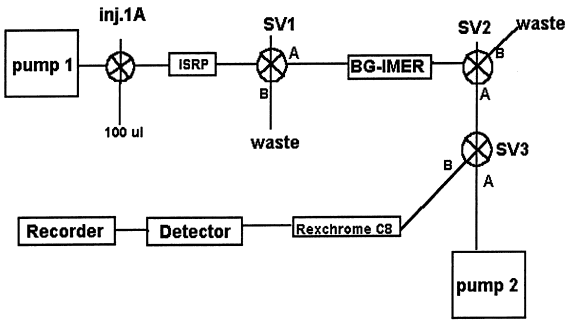

Fig. 2. A schematic diagram of the experimental procedure for off-line/on-line injections.

3. Results and discussion

3.1. Chromatography

Column chromatography has been proven to be a powerful tool for the separation and quantification of drugs in complex matrices of biomolecules. Complicated separation techniques are often necessary in order to isolate the desired drug molecule out of a biological sample (such as serum, plasma or urine). These involve off-line and on-line techniques which are a fast and efficient way of isolating components from biological matrices (such as urine). In this study, a covalently immobilized β-glucuronidase (BG-IMER) enzyme column was used on-line in a coupled-column HPLC system to deconjugate a target glucuronide substrate, chloramphenicol-β-D-glucuronide. This substrate was chosen as an example to show the application of the BG-IMER column in the deconjugation of the glucuronide to its aglycone from a biological sample.

In this study, a series of experiments were conducted to select stationary and mobile phases that gave the optimum conditions for resolution and selectivity between hydrolyzed and non-hydrolyzed glucuronide products as well as endogenous con-

taminants in the urine samples. Initially, it was necessary to identify and differentiate both chloramphenicol and its glucuronide metabolite in the chromatograms (as shown in Fig. 3D), before using the BG-IMER enzyme column for deconjugation. Secondly, various columns were evaluated in their capacity to separate the target compounds from large hydrophilic peaks arising from the direct injection of urine samples. The desired separation could be accomplished on the IAM.PC and the ISRP stationary phases, using 50×4.6-mm I.D. columns. For this study we opted for the ISRP column, in part, because its outer hydrophilic surface prevents large biomolecules (proteins from urine) from accessing its inner layer and allows small molecules (analyte) to be retained and separated by the hydrophobic support [7].

The solvent used in the deconjugation of chloramphenicol-β-D-glucuronide, 0.01 *M* ammonium acetate, was used at a higher pH of 6.7 instead of our previously reported value of 5.0 [1] in order to provide a better separation between endogenous proteins found in urine and the compound (glucuronide) to be hydrolyzed. (β-Glucuronidase enzymes are reported to be stable from pH 5.0 to 7.5 [8].) The product (hydrolyzed glucuronide of chloramphenicol) eluting from the BG-IMER column was then concentrated on the bed of the C_8 column packing. A switching valve was then placed to position A, to separate both columns so that the products would be analyzed on the C_8 reversed-phase column using a gradient system from pump 2 and mobile phases A and B (refer to Section 2). Meanwhile, the BG-IMER column and the ISRP column were continually equilibrating with a mobile phase (pump 1) consisting of a 0.01 *M* ammonium acetate solution adjusted to pH 6.7 for the next injection.

3.2. Sample pretreatment and percent conversion

Urine blanks and standards were first centrifuged (12 000 *g*) for 5 min and then aliquots of the supernatant were injected into the ISRP column. Centrifugation was necessary since urine samples contain endogenous materials such as fibers that would eventually block the injection valve if they were directly injected.

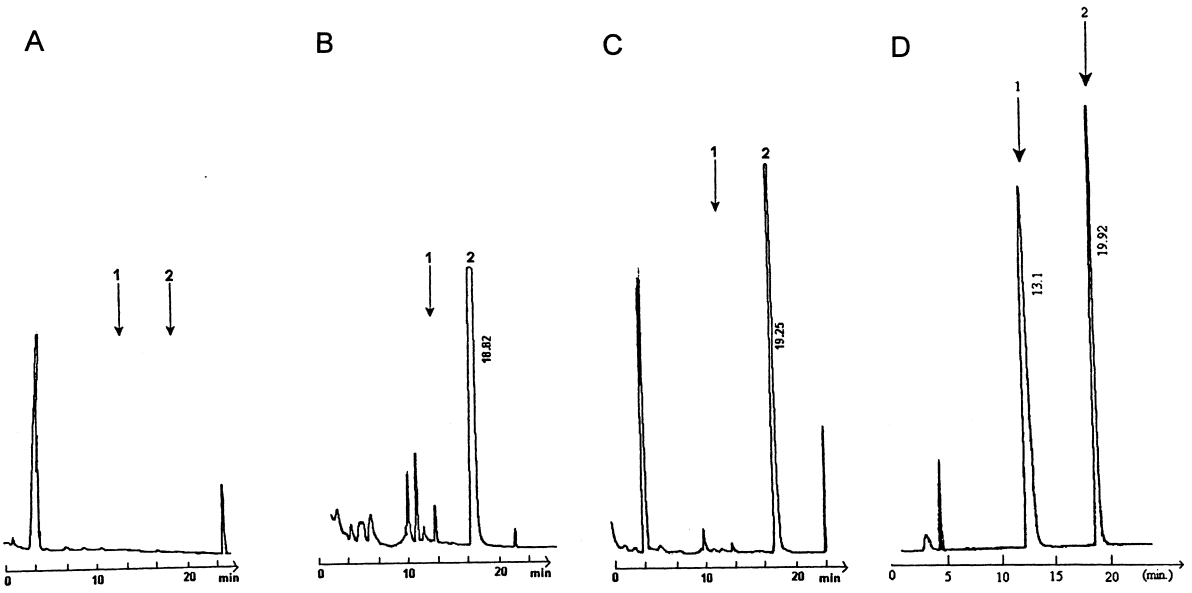

Fig. 3. Chromatograms representing (1) chloramphenicol-β-D-glucuronide; (2) chloramphenicol. (A) Blank urine sample with BG-IMER column; (B) urine sample spiked with the parent drug chloramphenicol; (C) calibration standard in urine (hydrolyzed chlor-g at 0.288 m*M*) with BG-IMER column; (D) control standards of chloramphenicol and its glucuronide in solution, without using the BG-IMER column.

We previously observed, in a concentration range of 0.02–2.0 m*M* (per 100-μl injection) from a solution of 0.01 *M* ammonium acetate, pH 5.0, a >98% conversion of the glucuronide to its aglycone by the BG-IMER column [1]. In this study, chloramphenicol-β-D-glucuronide was more than 98% converted to its parent drug (chloramphenicol) by the BG-IMER enzyme column at the concentration range of 0.07–1.1 m*M* (per 100-μl injection).

3.3. Specificity and selectivity

Blank urine (without compound) and control urine samples spiked with the aglycone drug (chloramphenicol) were injected into the coupled-column HPLC system. Analysis of the blank urine samples showed no interfering endogenous substances with the analysis of chloramphenicol (Fig. 3A). Any endogenous proteins interfering with the elution of the glucuronide metabolite by the BG-IMER column were resolved by increasing the pH of the mobile phase from 5.0 to 6.7, as well as discarding the eluted mobile phase from column A to waste for 5 min and then switching on-line to columns B and C. When passed on-line through the BG-IMER column, urine samples spiked with chloramphenicol (positive control) were associated with a peak at a retention time of 18.8 min (Fig. 3B) similar to the peak observed from the analysis of hydrolyzed glucuronide in urine samples (retention time of approximately 19.2 and 19.1 min (Fig. 3C and Fig. 4B)). To further validate the retention times of both chloramphenicol and its glucuronide, control blanks and standards were injected without using the BG-IMER enzyme column. The samples injected were from stock solutions, diluted with the initial mobile phase of 0.01 *M* ammonium acetate (pH 6.7) and urine samples spiked with chloramphenicol-β-D-glucuronide. Fig. 3D demonstrates that both the parent drug chloramphenicol and its glucuronide were well resolved on the chromatograms with a separation factor (α) of 1.52 and retention times of 19.9 and 13.1 min, respectively. The chloramphenicol-β-D-glucuronide spiked in urine eluted at 12.8 min when it was not injected on-line through the BG-IMER enzyme column and at 19.1 min when it was injected on-line with the enzyme column (Fig. 4).

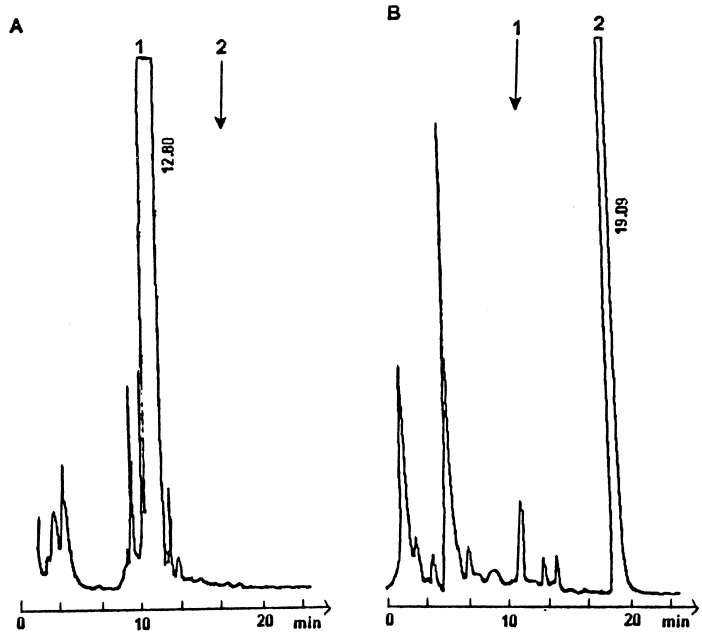

Fig. 4. Chromatograms representing chloramphenicol-β-D-glucuronide spiked in urine at 1.4 m*M*: (A) without the BG-IMER column ((1) chloramphenicol-β-D-glucuronide) and (B) with the BG-IMER column ((2) chloramphenicol).

3.4. Calibration curve

The standard calibration curve using urine samples spiked with chloramphenicol (obtained via the hydrolysis of the β-D-glucuronide) resulted in a correlation coefficient (R) of 0.983 (SYSTAT V 5.03 for Windows, Evanston IL, USA). This value is acceptable considering that no internal standard was used to correct for slight instrumental and procedure variations. With an internal standard, somewhat higher correlation coefficient values ($R=0.999$) have been reported, when glucuronides were deconjugated with β-glucuronidase solutions [9,10].

The assay was linear and consistent over the calibrated range of 0.36–5.7 mmol/ml. The inter- and intra-day precision of the analytical method ranged from 3 to 9% for the low concentrations (0.36 mmol/ml) and high concentrations (5.7 mmol/ml), respectively. The inter- and intra-day relative standard deviation (R.S.D.) for the accuracy of the analytical method at the lower and higher concentrations ranged from 5 to 15%. We believe that these numbers may be improved with the addition of an internal standard. The calculated intercept and slope were found to be 1030.3 area and 2771.8 area/mM, respectively.

4. Conclusion

A coupled-column HPLC system for analyzing chloramphenicol in urine samples has been developed by hydrolyzing glucuronide metabolites (chloramphenicol-β-D-glucuronide) with a novel reactor column. This immobilized enzyme reactor column, the 'BG-IMER', contains covalently bonded β-glucuronidase enzymes on a silica support packing and is used to hydrolyze glucuronide metabolites by on-line HPLC systems. This novel method of coupled-column chromatography for substrate deconjugation is considerably easier to perform than the conventional method using β-glucuronidase solutions.

Acknowledgements

We would like to thank Dr Terry Bowlin and BioChem Therapeutics Inc. for supporting this project.

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